Evidence for the occurrence of sibling species in *Eubazus* spp. (Hymenoptera: Braconidae), parasitoids of *Pissodes* spp. weevils (Coleoptera: Curculionidae)

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Abstract

Comparative studies were made on three presumed sibling species of the genus *Eubazus*, parasitoids of European *Pissodes* spp. weevils, to clarify their taxonomy and define diagnostic characters. Several populations of *E. semirugosus* (Nees), *E. robustus* (Ratzeburg) and *Eubazus* sp. were compared with respect to their morphology (mainly through morphometric analyses), fecundity, isoenzyme patterns and host preference. Crosses were made to assess the genetic and behavioural compatibility of the populations. In addition, the North American *E. crassigaster* (Provancher), a parasitoid of *Pissodes strobi* (Peck), was compared to *E. semirugosus*, a species selected for introduction against *P. strobi* in Canada. The ratio of the length of the ovipositor sheath to the fore wing length was the most discriminating morphometric variable, but discriminant analyses including several measurements were needed to completely separate European species. A canonical discriminant function provided a total separation between males of *E. crassigaster* and *E. semirugosus*, but not between females. *Eubazus crassigaster* and *E. semirugosus* were totally separated by the banding pattern of the enzyme phosphogluconate dehydrogenase whereas hexokinase and esterase provided a diagnostic separation between *Eubazus* sp. and *E. robustus*. *Eubazus* sp. differed from all the other species by having a greater number of ovarioles and, consequently, a higher potential fecundity. In a two-choice oviposition test, *E. semirugosus* and *Eubazus* sp. showed a significant preference for their natural host, *P. castaneus* De Geer and *P. piceae* (Illiger), respectively. A similar test made with their progenies reared under standard conditions showed that the difference in host preference was genetically fixed. Males and females of different species did not mate readily, in contrast to individuals from the same species. All attempts to interbreed *E. robustus* and *Eubazus* sp. failed, but a few crosses between *E. semirugosus* and the two other European species produced fertile offspring. These observations strongly suggest that the complex of *Eubazus* spp. parasitoids attacking *Pissodes* spp. in Europe is composed of at least three sibling species, two of which appear to have specialized on distinct host species that occupy exclusive microhabitats.

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Introduction

There is general agreement as to the importance of biosystematic studies on natural enemies in classical biological control (e.g. Caltagirone, 1985; Gauld, 1986; Powell & Walton, 1989; DeBach & Rosen, 1991). Selecting the most appropriate species or biotype for introduction against a pest is often considered an essential component in a biological control programme (Diehl & Bush, 1984; Ehler 1990; Hopper *et al.*, 1993). Unfortunately, parasitic Hymenoptera, the most frequently used biological control agents, are among the most difficult insects to identify and classify. Even in the well-studied western Palaearctic region, many groups of parasitic Hymenoptera need taxonomic revision, as some described species represent complexes of sibling or sister species while others, on further examination, lead to synonomies. Because of the frequent occurrence of sibling species and intraspecific variability, classical morphological criteria are often insufficient to clarify the taxonomy of parasitic Hymenoptera, and additional observations on quantitative morphometrics and comparative studies on genetic, ecological and behavioural differences are needed (Gauld, 1986; Powell & Walton, 1989).

Species of *Eubazus* (Hymenoptera: Braconidae) are egg–prepupal parasitoids of *Pissodes* spp. weevils (Coleoptera: Curculionidae), whose larvae live in trunks and cones of conifers. In Europe, there has been some confusion in the taxonomy of this parasitoid genus. In the past, several names were used for parasitoids of *Pissodes* spp.: *semirugosus* Nees, *atricornis* Ratzeburg, *firmus* Ratzeburg, *robustus* Ratzeburg and *mucronatus* Thunberg. These have been included in the genera *Allodorus* Foerster, *Brachistes* Wesmael, *Calyptus* Haliday, *Eubadizon* Nees, and *Sigalphus* Latreille (Ratzeburg, 1844, 1848; Marshall, 1888, 1891; Laidlaw, 1933; Fahringer, 1934). The more recent literature, however, has considered that only a single parasitoid species of this group attacks all *Pissodes* hosts in Europe (Haeselbarth, 1962; Annila, 1975; Roques, 1975; Alauzet, 1982; Mills & Fisher, 1986), but there is no agreement on either the generic or specific nomenclature. In a recent revision of the genera of the subtribe Brachistina, van Achterberg (1990) collapsed the former genera *Allodorus*, *Brachistes*, *Calyptus* and *Eubazus* Nees to sub-genera of the genus *Eubazus* s.l. According to his key, all the *Eubazus* species parasitizing *Pissodes* hosts belong to the sub-genus *Allodorus*. This genus, however, is still in need of revision, which is currently being undertaken by C. van Achterberg, Leiden. The *Eubazus* spp., parasitoids of *Pissodes* spp., will be revised in a forthcoming paper by C. van Acterberg and M. Kenis.

Recently, parasitoids of European *Pissodes* spp. were surveyed (Mills & Fisher, 1986; Kenis & Mills, 1994) as part of a biological control programme against the white pine weevil, *Pissodes strobi* (Peck), a native pest of pines and spruces in North America. For each of the five host species investigated, a *Eubazus* species was found to be the most abundant parasitoid. It quickly appeared from morphological and ecological observations that several species were probably involved. Until further investigations on the taxonomy of the genus are made, C. van Achterberg (personal communication) proposed separation of the European *Eubazus* parasitoids of *Pissodes* hosts into three species living in different microhabitats (Kenis & Mills, 1994). The species attacking *P. validirostris* (Sahlberg) in pine cones is considered to be *E. robustus* (Ratzeburg). *E. semirugosus* (Nees) (=*atricornis* Ratzeburg) is supposed to be the species parasitizing *P. castaneus* De Geer (=*notatus* (Fabricius).), *P. pini* (Linnaeus.) and *P. piniphilus* (Herbst) in pine trunks, while the species attacking *P. piceae* (Illiger) in fir trunks is regarded as an undescribed species, to be described in van Achterberg and Kenis (in preparation). Lovaszy (1941) has also reported a *Eubazus* species from the rare *P. harcyniae* (Herbst) in spruce trunks, but the identity of the parasitoid remains uncertain.

We have previously reported differences in the phenology of the *Eubazus* populations, reared under standard conditions, which support the separation of three distinct species (Kenis *et al.*, 1996). This study also identified a high altitude biotype of *E. semirugosus*, differing from other *Eubazus* populations by having an obligatory diapause, as the best candidate for introduction against *P. strobi* in North America due to synchronization of its phenology with that of the target host. A similar diapausing biotype of *E. robustus* was also found during this study but it is not yet clear whether these mountain, diapausing, populations belong to the same species as the lowland, non-diapausing populations.

Here we present comparative observations on the morphology, fecundity, isoenzyme composition, host preference and cross-matings of populations of *E. semirugosus*, *E. robustus* and *Eubazus* sp. When possible, investigations also included *Eubazus* (=*Allodorus*) *crassigaster* Provancher, a North American parasitoid of *P. strobi*, and *Eubazus* parasitoids from *P. harcyniae*. *Eubazus crassigaster* is morphologically very similar to the European species and, as *E. semirugosus* is likely to be introduced into North America for the control of *P. strobi*, it is of primary importance to define a clear method to distinguish between the European and the American species.

Material and methods

Collection and rearing

Several populations of *Pissodes* hosts and their respective *Eubazus* parasitoids were obtained from field collected material as detailed in Kenis & Mills (1994). *Eubazus semirugosus* was reared from *P. castaneus*, *P. pini* and *P. piniphilus* in pine trunks, *E. robustus* was obtained from *P. validirostris* in pine cones, and *Eubazus* sp. was reared from *P. piceae* in fir trunks. Two populations of a *Eubazus* species emerged from *P. castaneus* and *P. piniphilus* were suspected to be *E. robustus* because they emerged later from their hosts and they showed a longer development time than *E. semirugosus* when reared on *P. castaneus* in the laboratory (Kenis *et al.*, 1996). *Eubazus crassigaster* was reared from Sitka spruce leaders in British Columbia, Canada, by M.A. Hulme, Pacific Forestry Centre, Victoria, British Columbia, Canada and sent as adults to the European Station of the International Institute of Biological Control at Delémont, Switzerland. In addition, 15 pinned females of a *Eubazus* species obtained from *P. harcyniae* in spruce trunks were received from W. Grodzky, Forest Research Institute, Krakow (Poland), and added to our morphometric analyses. Site and collection characteristics are described in table 1.

able 1. Characteristics of the collections sites, length of the ovipositor sheath (OVIP) and ratio of length of the ovipositor sheath to fore ing length (OVIP/WL) measured in females of *Eubazus* spp. from different populations.

ource host	Site (region, country[1], elevation)	Host tree	Measurements ± S.E. (min–max)		
			N	OVIP (10^{-2} mm)	OVIP/WL (×1000)
. semirugosus					
Pissodes castaneus	Beaumotte (Haute-Saône, F, 300 m)	*Pinus sylvestris*	20	194 ± 2 e,f,g (170–206)	459 ± 5 b (424–520)
P. castaneus	Fontainebleau (Seine-et-Marne, F, 100 m)	*P. sylvestris*	20	177 ± 4 c,d,e (154–218)	470 ± 4 b (432–507)
P. castaneus	Lorris (Loret, F. 100 m)	*P. nigra*	20	186 ± 2 c,d,e,f (161–204)	454 ± 5 b (414–488)
P. castaneus	Paimpont (Ille-et-Vilaine, F, 150 m)	*P. sylvestris*	20	170 ± 3 b,c (139–197)	457 ± 7 b (399–503)
P. castaneus	De Koog (Texel Isl., NL, 10 m)	*P. nigra*	20	199 ± 2 f,g (182–214)	457 ± 4 b (430–486)
P. castaneus	Thetford (Norfolk, GB, 50 m)	*P. sylvestris*	9	188 ± 5 (163–202)	475 ± 9 (440–511)
P. pini	Delémont (Jura, CH, 500 m)	*P. sylvestris*	20	183 ± 3 c,d,e,f (156–199)	466 ± 5 b (414–503)
P. pini	Zernez[2] (Graubűnden, CH, 1900 m)	*P. uncinata*	20	199 ± 3 f,g (180–221)	461 ± 5 b (409–496)
P. pini	Val-d'Ajol (Vosges, F, 550 m)	*P. sylvestris*	13	188 ± 4 c,d,e,f,g (156–204)	456 ± 7 b (423–508)
P. pini	Brusson[2] (Valle D'Aosta, I, 1500 m)	*P. sylvestris*	12	210 ± 3 g (197–230)	461 ± 7 b (430–509)
P. piniphilus	Grissheim (Baden-Wűttemberg, D, 200 m)	*P. sylvestris*	20	188 ± 3 c,d,e,f,g (149–206)	461 ± 6 b (402–521)
. robustus					
P. validirostris	St-Crépin (Hautes-Alpes, F, 900 m)	*P. sylvestris*	20	144 ± 2 a (130–161)	376 ± 5 a (334–439)
P. validirostris	Fontainebleau (Seine-et-Marne, F, 100 m)	*P. sylvestris*	20	148 ± 1 a (139–156)	370 ± 3 a (342–390)
P. validirostris	Névache[2] (Hautes-Alpes, F, 1700 m)	*P. uncinata*	20	146 ± 2 a (120–161)	366 ± 5 a (300–414)
P. validirostris	Leuk (Valais, CH, 900 m)	*P. sylvestris*	20	145 ± 1 a (134–151)	373 ± 3 a (345–407)
P. validirostris	Olivet (Loiret, F, 100 m)	*P. nigra*	20	152 ± 1 a,b (144–163)	380 ± 3 a (352–409)
P. validirostris	Sacele (Transilvania, ROM, 700 m)	*P. sylvestris*	20	145 ± 1 a (139–154)	378 ± 4 a (350–420)
ubazus sp.					
P. piceae	Val-d'Ajol (Vosges, F, 550 m)	*Abies alba*	20	271 ± 3 h (249–290)	540 ± 5 c (490–571)
P. piceae	Leuk-Albinen (Valais, CH, 1250 m)	*A. alba*	20	260 ± 3 h (232–278)	526 ± 4 c (500–554)
P. piceae	Delémont (Jura, CH, 500 m)	*A. alba*	5	271 ± 5 (255–284)	536 ± 8 (517–557)
. crassigaster					
P. strobi	Port McNeill (Vancouver Isl., CAN, 20 m)	*Picea sitchensis*	20	171 ± 3 b,c,d (139–192)	437 ± 4 b (409–478)
P. strobi	Fair Harbour (Vancouver Isl., CAN, 20 m)	*P. sitchensis*	20	178 ± 2 c,d,e (156–192)	443 ± 4 b (418–485)
Jncertain species					
P. harcyniae	Szklarska Poreba (Sudety Mts., POL, 800 m)	*P. abies*	15	192 ± 4 d,e,f,g (168–223)	464 ± 9 b (394–522)
P. piniphilus	Delémont[3] (Jura, CH, 500 m)	*Pinus sylvestris*	20	141 ± 1 a (130–156)	387 ± 4 a (357–414)
P. castaneus	Fontainebleau[3] (Seine-et-Marne, F, 100 m)	*P. sylvestris*	9	153 ± 1 (149–156)	383 ± 6 (358–408)

CAN, Canada; CH, Switzerland; D, Germany; F, France; GB, Great Britain; I, Italy; NL, The Netherlands; POL, Poland; ROM, Romania.
Diapausing biotypes of *E. semirugosus* and *E. robustus*.
Slow developing populations.
Equal letters in columns indicate homogeneous groups at 0.05 significance level, Scheffe multiple range test. Sample sizes of less than 0 were not tested statistically.

Table 2. Characters selected for measurements on 20 males (M) and/or 20 females (F) of 17 *Eubazus* spp. populations.

Abbreviation	Measurement	Sex
AL3	Length of 3rd antennal segment	M
MSH	Mesosoma height	M F
MSL	Mesosoma length	M F
OVIP	Length of ovipositor sheath	F
T2	Length of 2nd tergite	M F
T3	Length of 3rd tergite	M F
T4	Apparent length of 4th tergite in dorsal view	M
T5–7	Apparent length of tergites 5 to 7 in dorsal view	M
S1	Length of 1st abdominal suture	M F
S2	Length of 2nd abdominal suture	M
FL	Length of hind femur	M F
FW	Width of hind femur	F
TL	Length of hind tibia	M F
TW	Width of hind tibia	F
BTL	Length of hind basitarsus	M F
BTW	Width of hind basitarsus	F
WL	Fore wing length	M F
W3SR	Vein 3-SR + SR1 of fore wing	M
HH	Height of head	M
OOL	Ocullar–ocellar line	M F
POL	Postocellar line	M F

Unless otherwise mentioned, all parasitoid material used in this study emerged from field collected material.

Eubazus parasitoids and *P. castaneus* were reared in the laboratory as described in Kenis (1994). *Pissodes piceae* was reared using the same methods as for *P. castaneus*, except that the rearing host was silver fir, *Abies alba*, and adults were collected in the field rather than reared from collected trunks.

Morphometric analyses

A preliminary analysis was made of 43 morphometric parameters from five male and five female specimens from five *Eubazus* populations: *E. semirugosus* from *P. castaneus* (collected at Beaumotte) and from *P. pini* (Zernez), *E. robustus* from *P. validirostris* (St.-Crépin), *Eubazus* sp. from *P. piceae* (Val-d'Ajol), and *E. crassigaster* from *P. strobi* (Port McNeill). The morphometric parameters were selected as those most commonly used in taxonomic studies of the Braconidae and were measured according to van Achterberg (1988), except the ocular–ocellar line which we defined as the shortest distance between the posterior ocellus and eyes.

Although commonly used in insect morphometric analyses, ratios are sometimes criticized (e.g. Reyment *et al.*, 1984). However, as insect parasitoids vary in absolute size according to the quality of the host species (e.g. Kishi, 1970; Mendel, 1986), all 43 morphometric measurements were divided by the hind femur length and the fore wing length to provide two sets of ratios for preliminary analysis. Additionally, ratios commonly used in braconid taxonomy were calculated (e.g. van Achterberg, 1990). All ratios were analysed using a one-way ANOVA. Measurements of ratios that exhibited a significant level of variation between *Eubazus* spp. populations ($P < 0.01$) were selected for further study (table 2). Each of these characters were measured on 20 male and 20 female parasitoids from 17 *Eubazus* populations. The same ratios were calculated and analysed using a one-way ANOVA. Finally, the most discriminating ratios were measured on an additional eight *Eubazus* populations. When ratios failed to provide a complete separation between *Eubazus* spp., discriminant analyses were performed on the individual measurements and morphometric characters were compounded into canonical discriminant functions using the SPSS (1993) procedure.

Five populations, one of each of the four presumed *Eubazus* spp. (three Palaearctic and one Nearctic) plus a population of the diapausing biotype of *E. semirugosus*, were reared on *P. castaneus* for one generation and specimens of the F1 generation were compared using morphometric measurements found most discriminating in the parent generation.

In addition to morphometric analyses, *Eubazus* parasitoids were carefully examined for other non-morphometric taxonomic characters.

Specimens of *Eubazus* spp. used in this study are maintained in the collections of the IIBC European Station in Delémont, Switzerland, and the Nationaal Natuurhistorisch Museum in Leiden, The Netherlands.

Fecundity

The potential fecundity of the *Eubazus* parasitoids was compared by counting the number of ovarioles per female, a trait which has been shown to be closely correlated with the number of eggs available for oviposition (Price, 1975). Between 5 and 41 females from several populations of *E. semirugosus*, *E. robustus*, *Eubazus* sp. and *E. crassigaster* were dissected and the number of ovarioles was counted. For each female, the hind femur length was measured as an indication of adult size.

Isoenzyme analyses

Six *Eubazus* populations were compared using isoenzyme electrophoresis: *E. semirugosus* from *P. pini* collected at Delémont and Zernez, *E. robustus* from *P. validirostris* at St. Crépin and Fontainebleau, *Eubazus* sp. from *P. piceae*

at Val-d'Ajol and *E. crassigaster* from *P. strobi* at Fair Harbour.

Horizontal starch gel electrophoresis was performed using the standard procedures and modified protocols from Murphy *et al.* (1990). Five males and five females from each population were assayed for 13 enzyme systems. These were: aspartame aminotransferase (AAT), catalase (CAT), cytosol aminopeptidase (CAP), esterase (EST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase (HK), l-iditol dehydrogenase (IIDH), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucomutase (PGM), phosphogluconate dehydrogenase (PGDH), trehalase (TRE), and xantine dehydrogenase (XDH). Of these, six could not be stained, or staining was not clear enough for reliable interpretation (CAT, CAP, GAPDH, IIDH, TRE and XDH) whilst three loci stained and resolved well but showed no difference in band pattern (AAT, ME and MDH-2). Five loci (EST, HK, MDH-1, PGDH and PGM) showed variation between *Eubazus* populations and were tested on 18 to 50 specimens from the same populations.

Given the small number of populations and enzyme systems studied, no serious attempt was made to study the genetic variation between populations. Instead, the isoenzyme analyses were performed to estimate their potential as identification tools for *Eubazus* spp.

Cross-mating experiments

Males and females of different *Eubazus* populations and biotypes were crossed to assess their genetic compatibility. One to three virgin females less than 1 day old were placed in 5.5×2 cm vials with males that were 0–10 days old. For each experiment, the sex ratio was one female to two males. The mating behaviour was observed in the vial for 30 min and copulations were monitored. Then, the insects were put in 50×30×30 cm gauze covered wooden cages for further matings. Two days later, Scots pine logs containing fresh eggs of *P. castaneus* were offered to the females for oviposition over a period of 2 days. The logs were then kept at 23°C and the emergence of male and female parasitoids was monitored daily. At 23°C, *Eubazus* spp. emerged between 30 and 75 days after oviposition. After the emergence period, logs from crosses involving the diapausing biotype of *E. semirugosus* were placed in a humid chamber at 2°C for three months and incubated again for parasitoid emergence. To assess the fertility of the female offspring, these were mated with their brothers and reared for a third generation. Rates of diapause observed in male and female offspring from crosses between the diapausing and the non-diapausing biotypes of *E. semirugosus* were compared with the rates of diapause observed in laboratory rearing of both biotypes as described by Kenis *et al.* (1996).

Host preference

Only two host species, *P. castaneus* and *P. piceae*, were reared in the laboratory because it was too difficult to rear *P. validirostris* in pine cones and *P. strobi* could not be reared in Europe because of the quarantine risk. Thus host preference investigations were restricted to two parasitoid species, *E. semirugosus* from *P. pini*, collected at Delémont, and *Eubazus* sp. from *P. piceae*, collected at Val-d'Ajol.

A cut section (*c.* 30×7 cm) of a trunk of *Pinus sylvestris* Linnaeus and one of *Abies alba* Miller (Pinaceae), containing a minimum of 20 eggs of *P. castaneus* and *P. piceae*, respectively, were placed vertically in opposite corners of a gauze covered wooden cage (50×30×30 cm). A naive, mated, female parasitoid, at least 2 days old, was placed in the middle of the cage and allowed to select one of the two hosts. The first oviposition attempt was monitored. Then the female was removed from the cage and the logs were swapped. For both species, an F1 generation was reared on *P. castaneus* and the female offspring were tested using the same procedure as for the parent generation. Between 18 and 49 females per species and per generation were tested.

Results

Morphometrics

Females

Several measurements and ratios provided significant differences between *Eubazus* spp. when analysed by ANOVA. For most of them, however, groups largely overlapped. Only the length of the ovipositor sheath could be considered as a diagnostic character. Table 1 shows, for several populations of all *Eubazus* spp., the variation in the absolute length of the ovipositor sheath and in the ratio ovipositor sheath length to fore wing length (OVIP/WL), the fore wing length being used as an indicator of the body size. Similar results were obtained when the ovipositor was divided by other measurements, such as hind femur length, hind tibia length, or mesosoma length. The ovipositor is shorter for *E. robustus* and longer from *Eubazus* sp. There was no overlap in the ovipositor length between *Eubazus* sp. and the other species. The ratio OVIP/WL provided a discrete separation between *E. robustus* and *Eubazus* sp., and between *E. crassigaster* and *Eubazus* sp., but *E. semirugosus* tended to overlap with all other species, although the overlap with *E. robustus* and *Eubazus* sp. was weak. There was no significant geographic variation in the ratio OVIP/WL between populations from the same species, including the diapausing and the non-diapausing biotypes of *E. semirugosus* and *E. robustus*, while sympatric populations of different species were as different as allopatric populations. The two parasitoid populations that emerged from *P. castaneus* and *P. piniphilus*, which were suspected to be *E. robustus* because of their longer development time in comparison to *E. semirugosus*, indeed had similar OVIP/WL ratios to the populations of *E. robustus* from *P. validirostris*.

Differences in the OVIP/WL ratio were maintained when *Eubazus* species were reared under standard conditions on *P. castaneus* in the laboratory (table 3). Ratios appeared to be better taxonomic characters than absolutes sizes. Indeed, for all species there was no difference in the relative size of the ovipositor between the parent and the F1 generation, while the absolute size of the ovipositor of *Eubazus* sp. from *P. piceae* significantly decreased when reared on the smaller *P. castaneus*.

The two sympatric populations of *E. semirugosus* and *E. robustus* from Fontainebleau were totally separated using the ratio OVIP/WL. However, when all the populations of *E. semirugosus* and *E. robustus* were included in the analysis, a discrete separation between the two species was obtained only by performing a discriminant analysis including 15 morphometric characters (fig. 1). When the discriminant function was applied to the doubtful, 'late emerging'

Table 3. Morphometric measurements of parent and F1 generations in females of *Eubazus* spp. Parent generations (P) emerged from field collected hosts, F1 generations emerged from *Pissodes castaneus* in the laboratory.

Source host (collection site)		Measurements ± S.E. (min–max)		
		OVIP (10^{-2} mm)	WL (10^{-2} mm)	OVIP/WL (×1000)
E. semirugosus				
P. castaneus (Paimpont)	P	170 ± 3 b (139–197)	373 ± 7 a,b (319–435)	457 ± 7 b,c (399–503)
	F1	168 ± 2 b (144–180)	359 ± 7 a (302–412)	470 ± 5 c (432–517)
P. pini (Zernez)[1]	P	199 ± 3 d (180–221)	432 ± 7 d (383–487)	461 ± 5 b,c (409–496)
	F1	186 ± 2 c,d (158–204)	405 ± 7 b,c,d (336–458)	459 ± 5 b,c (431–515)
E. robustus				
P. validirostris (Fontainebleau)	P	148 ± 1 a (139–156)	401 ± 3 b,c,d (377–423)	370 ± 3 a (342–390)
	F1	146 ± 2 a (125–156)	377 ± 6 a,b (302–435)	387 ± 3 a (359–415)
Eubazus sp.				
P. piceae (Val-d'Ajol)	P	271 ± 3 f (249–290)	503 ± 6 e (447–563)	540 ± 5 d (490–571)
	F1	230 ± 3 e (194–254)	420 ± 8 c,d (348–464)	550 ± 5 d (502–610)
E. crassigaster				
P. strobi (Port McNeill)	P	171 ± 3 b,c (139–192)	391 ± 5 a,b,c (331–423)	437 ± 4 b (409–478)
	F1	175 ± 3 b,c (144–202)	403 ± 8 b,c,d (313–458)	436 ± 5 b (392–480)

[1]Diapausing biotype of *E. semirugosus*.
Equal letters in columns indicate homogeneous groups at 0.05 level. Scheffe multiple range test.
N = 20 for each population and generation.

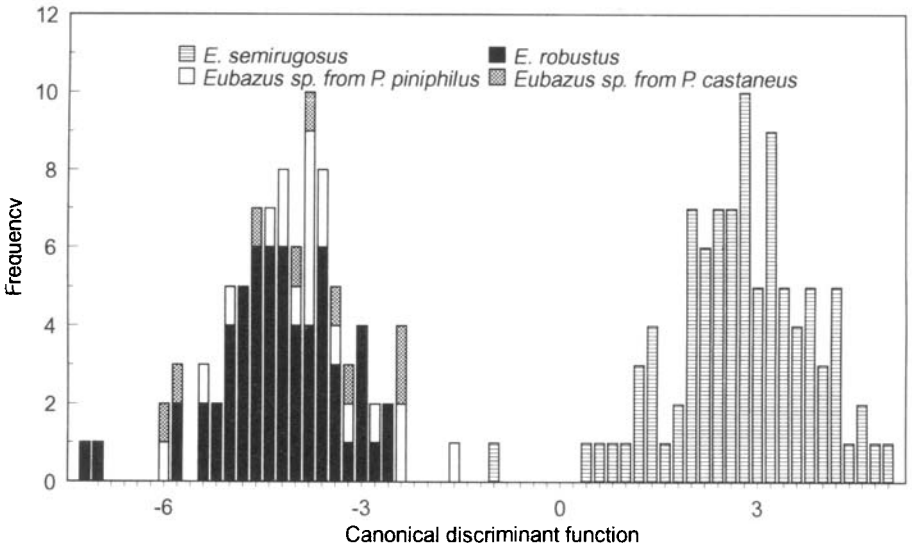

Fig. 1. Histogram of the canonical discriminant function for females of all populations of *Eubazus semirugosus* from *Pissodes pini* and *P. castaneus*, and *E. robustus* from *P. validirostris*. The same function was later applied to the doubtful, late emerging *Eubazus* sp. populations reared from *P. piniphilus* from Delémont, and *P. castaneus* from Fontainebleau. Function: –0.030 MSH + 0.126 OVIP – 0.011 S1 – 0.011 WL – 0.338 POL + 0.346 BTW + 0.097 FL + 0.004 TL + 0.114 TW – 0.025 OOL – 0.238 BTL – 0.087 FW + 0.104 T2 + 0.051 T3 – 0.050 MSL – 6.429.

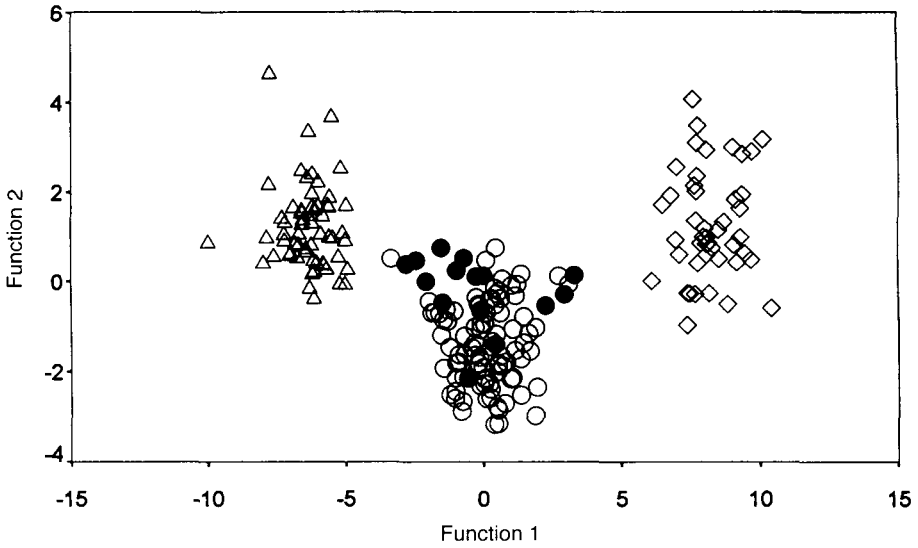

Fig. 2. Plots of first versus second canonical discriminant functions for females of all populations of *Eubazus semirugosus* (open circles) from *Pissodes castaneus* and *P. pini*, *E. robustus* (triangles) from *P. validirostris* and *Eubazus* sp. (squares) from *P. piceae*. The same two functions were later applied to the 15 undetermined specimens from *P. harcyniae* (solid circles). Function 1: –0.008 MSH – 0.024 MSL + 0.130 OVIP + 0.033 S1 – 0.002 T3 – 0.018 WL – 0.016 OOL – 0.393 POL – 0.155 BTL + 0.304 BTW + 0.086 FL – 0.085 FW – 0.025 TL + 0.014 TW + 0.061 T2 – 9.531; Function 2: +0.025 MSH + 0.038 MSL – 0.015 OVIP + 0.075 S1 – 0.087 T3 – 0.015 WL + 0.007 OOL + 0.093 POL + 0.223 BTL – 0.218 BTW + 0.095 FL – 0.091 FW – 0.123 TL – 0.044 TW – 0.032 T2 – 2.734.

populations from *P. piniphilus* and *P. castaneus*, these clearly clustered with the *E. robustus* group rather than with *E. semirugosus*.

The diapausing biotypes of *E. semirugosus* and *E. robustus* were morphologically identical to their respective non-diapausing biotype. For both species, not a single ratio was found significantly different between the two biotypes.

Figure 2 shows the graph of the two canonical discriminant functions that maximize the separation between the three European *Eubazus* spp. When plotted in this graph, the *Eubazus* population from *P. harcyniae* clearly clustered with *E. semirugosus*. In multiple range tests involving all *Eubazus* spp. populations, no significant difference was found in any ratio between the population from *P. harcyniae* and a *E. semirugosus* population.

Eubazus semirugosus and *E. crassigaster* largely overlapped for all ratios tested, and no discriminant analysis provided a complete separation between the females of these two species. The best result in separating *E. crassigaster* from *E. semirugosus* was achieved using a canonical discriminant

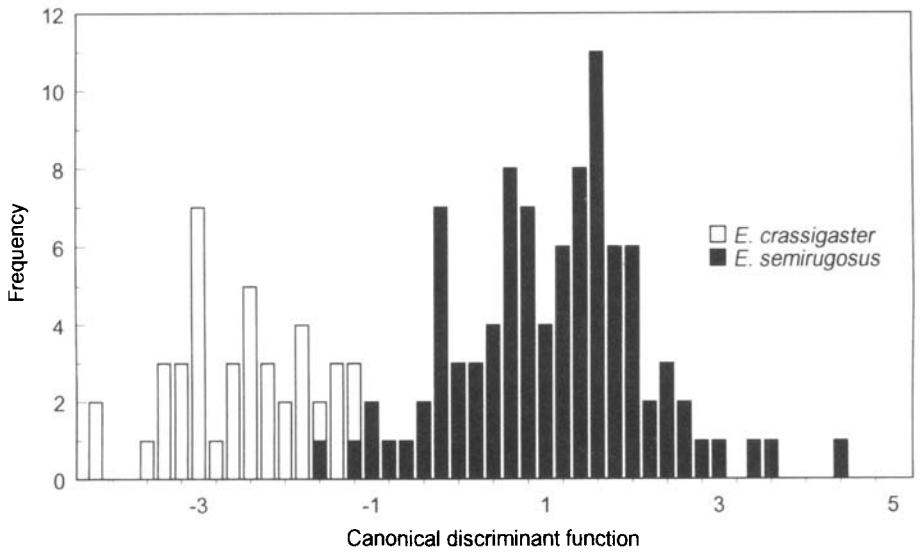

Fig. 3. Histogram of the canonical discriminant function for females of all populations of *Eubazus semirugosus* from *Pissodes pini* and *P. castaneus*, and *E. crassigaster* from *P. strobi*. Function: 0.129 OOL + 0.452 POL + 0.059 BTL – 0.102 BTW – 0.071 FL + 0.353 FW – 0.003 TL + 0.316 TW – 0.121 T2 – 0.013 MSH – 0.044 MSL + 0.043 OVIP – 0.044 S1 – 0.050 T3 + 0.001 WL – 5.815.0.

Table 4. Most significant ratios observed in males of *Eubazus* spp. from different populations.

Host (site)	N	Ratios ± S.E. (min–max) × 100		
		AL3/BTL	S1/FL	OOL/POL
E. semirugosus				
Pissodes castaneus (Beaumotte)	20	58 ± 1 c,d,e,f (52–65)	68 ± 1 a,b (56–80)	159 ± 3 a,b,c,d,e,f (141–179)
P. castaneus (Fontainebleau)	12	58 ± 1 b,c,d,e,f (50–64)	61 ± 1 a (57–69)	167 ± 3 c,d,e,f,g (154–183)
P. castaneus (De Koog)	20	60 ± 1 e,f (56–65)	68 ± 1 a,b (63–73)	161 ± 2 b,c,d,e,f (143–183)
P. castaneus (Thetford)	7	61 ± 2 (55–67)	68 ± 2 (56–70)	152 ± 3 (140–160)
P. pini (Zernez)[1]	20	60 ± 1 d,e,f (54–68)	66 ± 1 a,b (58–78)	166 ± 3 e,f,g (144–192)
P. pini (Delémont)	20	60 ± 1 e,f (52–67)	66 ± 1 a,b (61–78)	163 ± 3 d,e,f (150–192)
P. pini (Brusson)[1]	12	62 ± 1 e,f (57–65)	65 ± 1 a,b (61–72)	165 ± 2 b,c,d,e,f,g (150–177)
P. pini (Val-d'Ajol)	7	58 ± 1 (54–61)	66 ± 1 (61–70)	162 ± 4 (146–175)
P. piniphilus (Grissheim)	20	58 ± 1 c,d,e,f (52–65)	62 ± 1 a (56–70)	167 ± 2 e,f,g (150–192)
E. robustus				
P. validirostris (St-Crépin)	20	56 ± 1 a,b,c,d,e (43–63)	78 ± 1 c,d (70–85)	148 ± 3 a,b,c,d,e (129–177)
P. validirostris (Fontainebleau)	20	56 ± 1 a,b,c,d,e (50–64)	79 ± 1 c,d (57–86)	140 ± 2 a (120–150)
P. validirostris (Nevache)[1]	20	55 ± 1 a,b,c,d (50–62)	82 ± 1 d,e (76–93)	146 ± 2 a,b,c,d (127–167)
P. validirostris (Leuk)	20	53 ± 1 a,b,c (43–59)	78 ± 1 c,d (75–83)	144 ± 2 a,b,c (129–157)
P. validirostris (Sacele)	15	56 ± 1 a,b,c,d,e (52–59)	85 ± 1 d,e (79–91)	142 ± 2 a,b (129–158)
Eubazus sp.				
P. piceae (Val-d'Ajol)	20	52 ± 1 a,b (44–57)	72 ± 1 b,c (60–89)	177 ± 4 f,g,h (153–221)
P. piceae (Leuk-Albinen)	17	54 ± 1 a,b,c,d (46–60)	71 ± 1 b,c (61–81)	174 ± 4 f,g,h (154–220)
P. piceae (Delémont)	4	46 ± 0 (45–46)	71 ± 1 (67–73)	182 ± 14 (150–217)
E. crassigaster				
P. strobi (Port McNeill)	20	63 ± 1 f (58–71)	86 ± 1 e (79–95)	192 ± 3 h (171–217)
P. strobi (Fair Harbour)	20	60 ± 1 e,f (52–67)	83 ± 1 d,e (76–87)	187 ± 3 g,h (167–200)
Uncertain species				
P. piniphilus (Delémont)[2]	20	52 ± 1 a (47–57)	73 ± 1 b,c (67–83)	146 ± 3 a,b,c,d (120–167)
P. castaneus (Fontainebleau)[2]	6	54 ± 1 (50–57)	90 ± 2 (83–98)	139 ± 4 (125–150)

[1]Diapausing biotypes of *E. semirugosus* and *E. robustus*.
[2]Slow developing populations.
Equal letters in columns indicate homogeneous groups at 0.05 level, Scheffe multiple range test.
Sample sizes of less than 10 were not tested statistically.

function including 15 characters, in which case 97% of the individuals were classified correctly (fig. 3).

Males

No single ratio of male measurements provided a discrete separation between *Eubazus* spp., but the ratio of first metasomal suture to hind femur length (S1/FL) was the most discriminating, with *E. semirugosus* having a conspicuously shorter relative suture length than *E. crassigaster* and *E. robustus* (table 4). *Eubazus crassigaster* was significantly different from *Eubazus* sp. and *E. robustus* in the ratio AL3/BTL and the OOL/POL ratio was significantly higher in *E. crassigaster* and *Eubazus* sp. than in *E. robustus*. The two doubtful, late emerging populations from *P. piniphilus* and *P. castaneus* had similar ratios to the populations of *E. robustus*.

As observed in females, the absolute size of *Eubazus* sp. decreased when reared on *P. castaneus* in the laboratory (table 5). In contrast, the S1/FL ratio was constant between the field and laboratory generation. Differences in the S1/FL

Table 5. Morphometric measurements of parent and F1 generations in males of *Eubazus* spp. Parent generations (P) emerged from field collected hosts, F1 generations emerged from *Pissodes castaneus* in the laboratory.

Source host (collection site)		Measurements ± S.E. (min–max)		
		FL (10^{-2} mm)	S1 (10^{-2} mm)	S1/FL (×100)
E. semirugosus				
P. castaneus (Beaumotte)	P	101 ± 2 b (94–118)	69 ± 2 b,c (58–91)	68 ± 1 a,b (56–80)
	F1	86 ± 2 a (72–94)	55 ± 1 a (41–62)	63 ± 1 a (57–70)
P. pini (Zernez)[1]	P	108 ± 2 b (82–125)	71 ± 2 b,c,d (55–86)	66 ± 1 a,b (58–78)
	F1	97 ± 2 a,b (74–113)	66 ± 2 a,b (50–84)	68 ± 1 a,b (62–74)
E. robustus				
P. validirostris (Fontainebleau)	P	102 ± 2 b (86–115)	80 ± 2 c,d,e (58–91)	79 ± 1 c,d (57–86)
	F1	100 ± 2 b (84–115)	81 ± 2 d,e (62–103)	81 ± 1 d,e (68–90)
Eubazus sp.				
P. piceae (Val-d'Ajol)	P	137 ± 2 c (118–154)	99 ± 2 f (79–115)	72 ± 2 b,c (60–89)
	F1	107 ± 2 b (86–122)	76 ± 2 b,c,d,e (65–89)	71 ± 1 b (66–78)
E. crassigaster				
P. strobi (Port McNeill)	P	100 ± 1 b (84–108)	87 ± 1 e (74–96)	86 ± 1 e (79–95)
	F1	97 ± 3 a,b (79–115)	83 ± 3 e (63–103)	86 ± 1 e (76–95)

[1]Diapausing biotype of *E. semirugosus*.
Equal letters in columns indicate homogeneous groups at 0.05 level, Scheffe multiple range test.
N = 20 for each population and generation.

ratio observed between *Eubazus* spp. in the parent generation were maintained in the laboratory generation reared on *P. castaneus*.

Two canonical functions maximizing the differences between European *Eubazus* spp. separated *Eubazus* sp. from *E. robustus* but *E. semirugosus* slightly overlapped with the

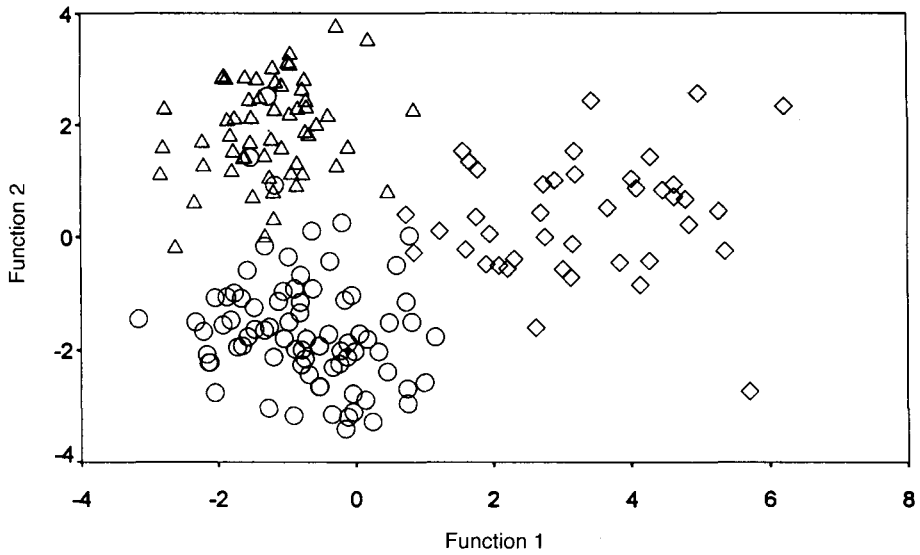

Fig. 4. Plots of first versus second canonical discriminant functions for males of all populations of *Eubazus semirugosus* (circles) from *Pissodes castaneus* and *P. pini*, *E. robustus* (triangles) from *P. validirostris* and *Eubazus* sp. (squares) from *P. piceae*. Function 1: 0.030 S2 – 0.001 T2 – 0.026 T3 – 0.258 OOL – 0.302 POL – 0.002 W3SR + 0.007 WL – 0.019 S1 – 0.011 MSL + 0.046 BTL + 0.058 FL + 0.003 TL – 0.020 HH + 0.089 MSH – 0.088 AL3 – 5.905; Function 2: 0.015 S2 – 0.134 T2 + 0.041 T3 – 0.247 OOL + 0.278 POL – 0.026 W3SR – 0.016 WL + 0.085 S1 + 0.076 MSL + 0.177 BTL + 0.012 FL – 0.064 TL – 0.070 HH – 0.091 AL3 + 2.589.

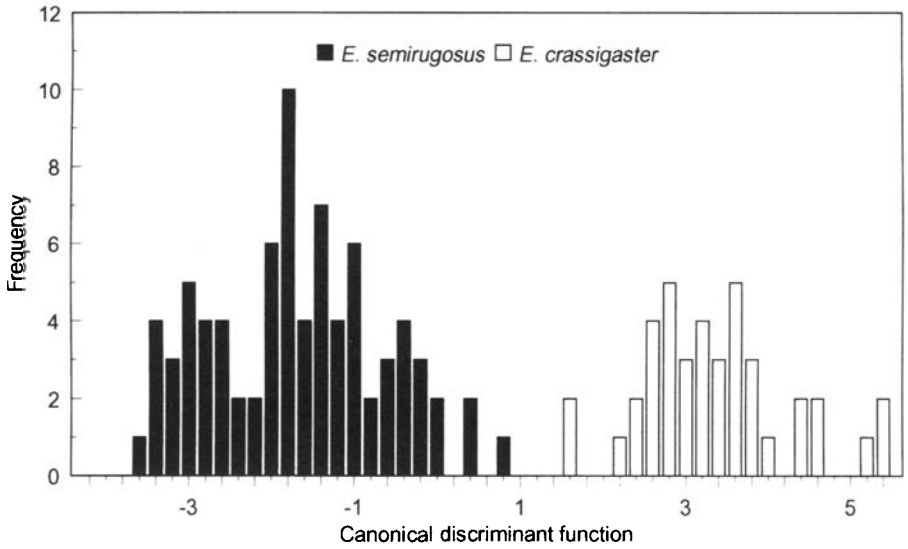

Fig. 5. Histogram of the canonical discriminant function for males of all populations of *Eubazus semirugosus* from *Pissodes pini* and *P. castaneus*, and *E. crassigaster* from *P. strobi*. Function: 0.009 S2 – 0.083 T2 + 0.023 T3 + 0.248 OOL – 0.699 POL + 0.057 W3SR – 0.023 WL + 0.180 S1 + 0.034 MSL – 0.025 BTL – 0.145 FL + 0.060 TL – 0.003 HH – 0.036 MSH – 0.038 AL3 + 2.056.

two other species (fig. 4). *E. crassigaster* and *E. semirugosus* were totally separated using a discriminant function including 15 morphometric characters (fig. 5).

An important variation was also found in the length of the fourth to seventh abdominal tergites. In *E. crassigaster* males, the apparent length of tergites five to seven is shorter than that of the fourth tergite, while it is longer in the European species. However, as the apparent length of the fourth to seventh abdominal tergites depends on the killing method, relative tergite lengths cannot be used as fixed taxonomic characters, but they certainly provide the easiest method for separating males of *E. crassigaster* and *E. semirugosus* when all specimens have been killed using the same method.

Non-morphometric variation

The only consistent morphological variation found among the *Eubazus* spp. is the clypeal tooth which, in both male and female *E. crassigaster*, is absent or reduced compared to the European species (fig. 6). However, in both groups there are some specimens that cannot be assigned with certainty to one of the two groups.

Fecundity

The four *Eubazus* spp. varied in the number of ovarioles per female (ANOVA: $F = 132.88$, d.f. = 3, $P < 0.001$). *Eubazus* sp. from *P. piceae* had almost twice as many ovarioles (mean = 38.0, S.D. = 4.4, min–max = 30–47, n = 41) as *E. semirugosus* (mean = 23.0, S.D. = 3.1, min–max = 11–28, n = 37), *E. robustus* (mean = 24.1, S.D. = 2.8, min–max = 18–29, n = 49) and *E. crassigaster* (mean = 21.4, S.D. = 1.8, min–max = 19–23, n = 5), suggesting a much greater fecundity. The number of ovarioles was correlated with the body size, estimated by the length of the hind femur, in *Eubazus* sp. ($r = 0.426$, n = 41, $P = 0.005$), but not in *E. semirugosus* ($r = -0.048$, n = 37, $P = 0.798$) or *E. robustus* ($r = 0.271$, n = 49, $P = 0.086$).

Isoenzyme analyses

Eubazus crassigaster was totally separated from all European *Eubazus* spp. by the banding pattern of the enzyme PGDH. It could also be separated from *E. robustus* by the banding pattern of HK and from *Eubazus* sp. by that of EST (table 6). Among the European species, HK and EST patterns

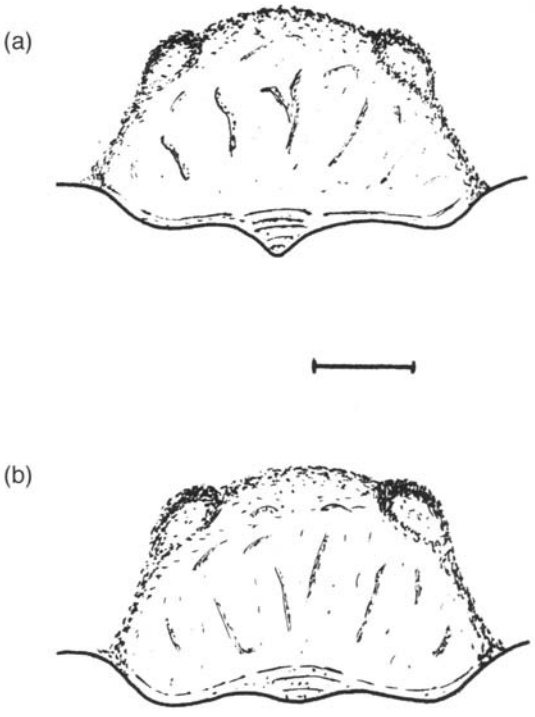

Fig. 6. Clypeus: (a) *Eubazus semirugosus*; (b) *E. crassigaster*; scale line = 0.1 mm.

Table 6. Allele frequenties for isoenzyme loci in populations of *Eubazus* spp.

Locus	Allele	*E. robustus*		*E. semirugosus*		*Eubazus* sp.	*E. crassigaster*
		St-Crépin	Fontainebleau	Zernez[1]	Delémont	Val-d'Ajol	Fair Harbour
HK	*n*[2]	*51*	*42*	*60*	*26*	*60*	*77*
	A	0.00	0.00	0.87	1.00	1.00	1.00
	B	1.00	1.00	0.13	0.00	0.00	0.00
MDH-1	*n*	*51*	*42*	*60*	*26*	*60*	*77*
	A	1.00	0.98	1.00	1.00	0.90	0.79
	B	0.00	0.02	0.00	0.00	0.00	0.01
	C	0.00	0.00	0.00	0.00	0.10	0.19
PGDH	*n*	*51*	*42*	*60*	*26*	*60*	*77*
	A	0.98	1.00	1.00	1.00	1.00	0.00
	B	0.00	0.00	0.00	0.00	0.00	1.00
	C	0.02	0.00	0.00	0.00	0.00	0.00
PGM	*n*	*51*	*42*	*60*	*26*	*60*	*77*
	A	0.00	0.00	0.00	0.00	0.00	0.01
	B	0.37	0.26	0.98	1.00	1.00	0.99
	C	0.59	0.71	0.00	0.00	0.00	0.00
	D	0.04	0.02	0.02	0.00	0.00	0.00
EST	*n*	*49*	*42*	0[3]	0[3]	*58*	*57*
	A	0.00	0.00			1.00	0.00
	B	0.00	0.00			0.00	0.12
	C	1.00	0.98			0.00	0.00
	D	0.00	0.02			0.00	0.88

[1]Diapausing biotype.
[2]Number of alleles scored (2×no. of females + no. of males).
[3]EST patterns did not resolve satisfactorily and are not included in the table.
Alphabetical order in allelic designations indicates rapidity of migration to the anode during electrophoresis (e.g. A migrates faster than B).

differentiated *E. robustus* from *Eubazus* sp., but *E. semirugosus* shared common HK bands with both species and EST could not be resolved satisfactorily for *E. semirugosus*. There was very little difference in the banding patterns between the diapausing and the non-diapausing biotypes of *E. semirugosus*, as well as between the two *E. robustus* populations tested.

Cross-mating experiments

The results of cross-mating studies are shown in table 7. In crosses between males and females of the same species, matings were usually observed within the first 5 min. Almost all these crosses gave rise to fertile females. In contrast, *Eubazus* males did not show much interest in females of other species. Only one interspecific mating was observed within the 30 min of observation, between a male of *E. semirugosus* and a female of *Eubazus* sp. However, one crossing between *E. semirugosus* and *Eubazus* sp. and two between *E. semirugosus* and *E. robustus* produced fertile females. All attempts to cross *E. robustus* and *Eubazus* sp. failed.

Most crosses between the diapausing and the non-diapausing biotypes of *E. semirugosus* resulted in fertile female offspring. When females from the non-diapausing biotype were crossed with males of the diapausing biotype, 69% of their female offspring entered into diapause while all males emerged. Crosses between females of the diapausing biotype and males of the non-diapausing biotype gave rise to all males and a majority of females entering into diapause (fig. 7). These results show that the diapause is genetically based and carried by both sexes.

Host preference

When given a choice for oviposition between eggs of *P. castaneus* and *P. piceae* in pine and fir logs, respectively, *E. semirugosus* females showed a strong preference for *P. castaneus* while females of *Eubazus* sp. from *P. piceae* significantly preferred their original host (fig. 8). When reared on *P. castaneus* for one generation, the offspring of *E. semirugosus* still preferred *P. castaneus*, but *Eubazus* sp. did not show a preference for either of the two proposed hosts. The difference in host preference between the two species was highly significant in the parent generation as well as in the F1 generation.

Discussion

According to Mayr's (1942) general definition, to which most systematists still adhere, species are groups of interbreeding populations that are reproductively isolated from one another. However, reproductive isolation in nature is not easy to verify. It is particularly difficult to determine whether two host-associated biotypes are just host races or sibling species, because host races are usually a step toward speciation and the limit between the two categories is rather arbitrary (Bush, 1975; Diehl & Bush, 1984). There are several examples in the literature showing that what were believed to be host races appeared to be distinct species after careful studies (e.g. Vet *et al.*, 1984; Holler, 1991). We consider, from the evidence provided, that the complex of *Eubazus* spp. parasitoids attacking *Pissodes* hosts in Europe is composed of at least three sibling species, each of them being largely specialized in a different microhabitat, as previously

suggested in earlier studies (Kenis & Mills, 1994; Kenis *et al.*, 1996). *Eubazus semirugosus* is found only on *P. castaneus*, *P. pini*, and *P. piniphilus* in pine trunks and, perhaps, on *P. harcyniae* in spruce trunks. *Eubazus* sp. is specific to *P. piceae* in fir trunks and *E. robustus* is primarily a parasitoid of *P. validirostris* in pine cones, but occasionally attacks *Pissodes* spp. in pine trunks.

Eubazus robustus and *Eubazus* sp. appear to be totally reproductively isolated, as all attempts to interbreed these two species failed, while successful reproduction was easily demonstrated within each species. Interbreeding between *E. semirugosus* and the two other European species is still possible, but difficult, apparently because adults of different species do not mate as readily as individuals from the same species. Preliminary studies on isoenzymes supported the idea of an intermediate position of *E. semirugosus*, as hexokinase banding patterns clearly separate *E. robustus* from *Eubazus* sp., while *E. semirugosus* shares bands of both species.

The ratio of ovipositor length to fore wing length provided the best morphometric character to separate the three European species, with *E. robustus* having the shortest, and *Eubazus* sp. the longest relative ovipositor length. Here again, *E. semirugosus* tended to overlap with both species, and only discriminant functions including several measurements provided a full separation between *E. semirugosus* and its two sibling species. The differences in morphology were not host-induced but genetically fixed as they were maintained when the *Eubazus* species were reared on a common host.

The intermediate position of *E. semirugosus* observed in cross-mating experiments, isoenzyme analyses and

Table 7. Results of crosses.

Species crossed[1] (m×f)	Collection sites (m×f)	Mating observed[2]	Offspring	
			in F1 (m/f)	in F2 (m/f)
Intrapopulation				
sem(c)×sem(c)	Beaumotte F×Beaumotte F	Y	14/8	0/7
rob×rob	St-Crépin F×St-Crépin F	Y	9/4	7/3
esp×esp	Val-d'Ajol F×Val-d'Ajol F	Y	10/12	9/6
Interpopulation				
sem (p)×sem(p)	Brusson CH×Val-d'Ajol F	Y	4/4	0/1
sem(p)×sem(c)	Zernez CH×Lorris F	Y	34/10	8/8
sem(p)×sem(p)	Zernez CH×Delémont CH	Y	25/21	NR[3]
sem(c)×sem(p)	Lorris F×Zernez CH	Y	13/23	6/8
sem(p)×sem(p)	Delémont CH×Zernez CH	Y	13/0	
sem(c)×sem(p)	Fontainebleau F×Zernez CH	Y	8/6	9/5
sem(c)×rob	Beaumotte F×Fontainebleau F	N	10/0	
sem(p)×rob	Delémont CH×Leuk F	N	19/0	
sem(c)×rob	Fontainebleau F×Fontainebleau F	N	10/0	
sem(p)×rob	Delémont CH×St-Crépin F	N	3/6	1/3
sem(c)×rob	Fontainebleau F×Leuk CH	N	9/0	
rob×sem(c)	Fontainebleau F×Beaumotte F	N	24/0	
rob×sem(c)	Fontainebleau F×Beaumotte F	N	33/0	
rob×sem(c)	Fontainbleau F×Beaumotte F	N	11/0	
rob×sem(p)	Leuk CH×Delémont CH	N	4/6	43/4
rob×sem(c)	Fontainebleau F×Fontainbleau F	N	16/0	
sem(p)×esp	Val-d'Ajol F×Val-d'Ajol F	N	15/0	
sem(p)×esp	Val-d'Ajol F×Val-d'Ajol F	Y	13/0	
sem(p)×esp	Zernez CH×Val-d'Ajol F	N	37/0	
sem(p)×esp	Delémont CH×Val-d'Ajol F	N	16/0	
esp×sem(p)	Val-d'Ajol F×Val-d'Ajol F	N	11/18	6/7
esp×sem(p)	Val-d'Ajol F×Delémont CH	N	41/0	
esp×sem(p)	Val-d'Ajol F×Zernez CH	N	10/0	
rob×esp	St-Crépin F×Val-d'Ajol F	N	26/0	
rob×esp	St-Crépin F×Val-d'Ajol F	N	10/0	
rob×esp	Fontainebleau F×Val-d'Ajol F	N	4/0	
esp×rob	Val-d'Ajol×St-Crépin F	N	14/0	
Unmated females				
sem(c)	Lorris F		12/0	
sem(p)	Val-d'Ajol F		12/0	
rob	Fontainebleau F		8/0	
esp	Val-d'Ajol F		18/0	

[1]sem(c), *Eubazus semirugosus* from *Pissodes castaneus*; sem(p), *E. semirugosus* from *P. pini*; sem(p), diapausing biotype of *E. semirugosus* from *P. pini*; rob, *E. robustus* from *P. validirostris*; Esp, *Eubazus* sp. from *P. piceae*.
[2]YES (Y), at least 1 mating observed during the first 30 minutes of contact; NO (N), no mating observed during the first 30 minutes of contact.
[3]NR, F1 not reared for a second generation.
Each row represents one cross test with 1 to 3 females and twice as many males as females.

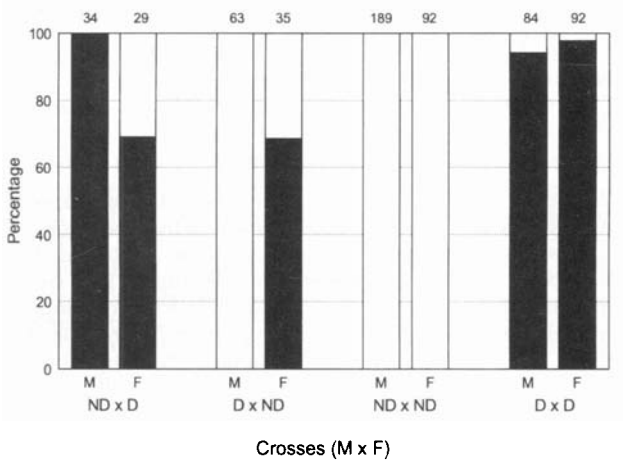

Fig. 7. Percentages of diapause (solid bars) and emergence (open bars) in male and female offspring resulting from crosses between the diapausing (D) and the non-diapausing (ND) biotypes of *Eubazus semirugosus*, and percentages of diapause and emergence observed in laboratory rearing of both biotypes. Numbers above the bars are sample sizes.

morphometric analyses suggests that *Eubazus* sp. and *E. robustus* have evolved separately from *E. semirugosus*, or from a common ancestor living in pine trunks. This hypothesis is supported by the fact that the majority of *Pissodes* species live in pine trunks (Kudela, 1974; O'Brien, 1989a,b). Very few species attack fir trunks and *P. validirostris* is the only *Pissodes* species living in cones.

Speciation is not complete among the European parasitoids, as *E. semirugosus* is still genetically compatible with the two other species when forced to cross in the laboratory. In the field, however, pre-mating barriers probably limit the possibilities for interbreeding. The most important factor favouring isolation is most certainly the fact that adults tend

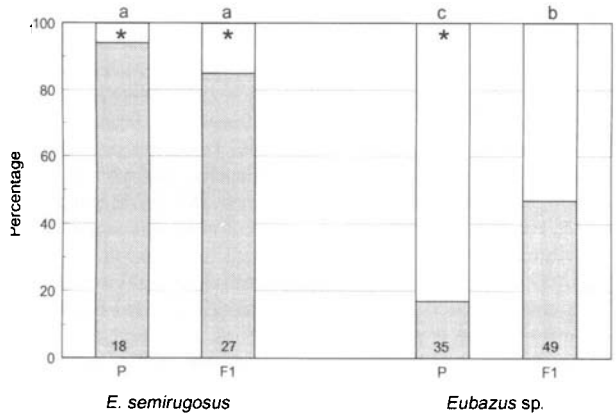

Fig. 8. Preference for first oviposition in *Eubazus semirugosus* (reared from *Pissodes pini*) and *Eubazus* sp. (reared from *P. piceae*) when given a choice between *P. castaneus* eggs in pine logs (hatched bars) and *P. piceae* eggs in fir logs (open bars). Parents (P) emerged from their original host while the F1 generation emerged from *P. castaneus* in rearing. Numbers in the bars are sample sizes. Stars in the bars indicate a significant preference for one of the two hosts (test for binomial distribution, $P < 0.05$). Different letters above two bars indicate a significant difference in host preference (2-tailed Fisher exact test, $P < 0.05$).

to mate with siblings at the emergence site, as observed in *Eubazus* sp. (Haeselbarth, 1962), *E. robustus* (Roques, 1975) and *E. semirugosus* (M. Kenis, unpublished data). We also observed that males show more interest in conspecific females than in females of other species. Other isolating factors include host preference, as shown with *E. semirugosus* and *Eubazus* sp., and temporal differences in the timing of adult emergence. Adult emergence periods overlap in the field, because *E. semirugosus* can emerge from April to October (M. Kenis, unpublished data), but the peak emergence periods are not synchronized, which reduces the possibility of interspecific mating. In the Swiss Jura, *E. semirugosus* has two peaks of emergence, in late April–early May and in July (Kenis *et al.*, 1996). Roques (1975) observed, in a similar climatic region in central France, that *E. robustus* emerges in late May and, to a lesser extent, in late August and in September, and Haeselbarth (1962) showed that in Bavaria *Eubazus* sp. mainly emerges from late May to late June.

Eubazus robustus was found to emerge from *P. castaneus* and *P. piniphilus* in pine trunks, and thus, mating with *E. semirugosus* could potentially occur between these two species. However, we observed that when *E. robustus* and *E. semirugosus* parasitize the same population of *P. castaneus*, *E. robustus* emerges later than *E. semirugosus*, which, again, limits the possibility of interbreeding (M. Kenis, unpublished data).

As shown by Annila (1975) and Roques (1975), about 25% of the *E. robustus* emerge from *P. validirostris* in late summer when there are no eggs of *P. validirostris* available for oviposition. Thus *P. castaneus*, *P. piniphilus* and perhaps *P. pini* can be used as alternate hosts for this late summer generation. *Pissodes piniphilus* appears to be a particularly suitable host for *E. robustus*. It is usually found in the crown of mature trees, i.e. in the vicinity of cones, while *P. castaneus* prefers to attack young trees and *P. pini* prefers dying or freshly cut trees. Furthermore, *P. piniphilus*, being the smallest *Pissodes* species, lays its eggs closer to the bark surface rendering them more accessible to *E. robustus* whose ovipositor is shorter than that of *E. semirugosus*. More generally, the length of the ovipositor in *Eubazus* spp. is related to the oviposition behaviour of their respective hosts. *Pissodes piceae* is the largest host and its long rostrum allows it to dig oviposition holes deep in the bark. Furthermore, it lays up to 15 eggs in the same hole (Haeselbarth, 1962), and thus, to reach all the eggs, *Eubazus* sp. needs a longer ovipositor than its sister species whose hosts lay eggs in groups of one to five and closer to the bark surface.

In the laboratory, *E. semirugosus* and *Eubazus* sp. preferred to oviposit in their respective hosts, and this difference was maintained in a second generation after rearing on *P. castaneus* under standard conditions, which suggests that host preference has a genetic basis. Nevertheless, for *Eubazus* sp., rearing on *P. castaneus* significantly increased the acceptability of this unnatural host compared to the parent generation originating from *P. piceae*, suggesting that host preference is also influenced by the host, or host habitat, experienced during the pre-emergence period, a phenomenon often observed in insect parasitoids (e.g. Kudon & Berisford, 1980; Turlings *et al.*, 1993).

Eubazus sp. differed from all the other species by having a greater number of ovarioles and, consequently, a higher potential fecundity. The variation observed in the fecundity of *Eubazus* spp. probably results from a variation in the

spatial distribution of their respective hosts. Most solitary parasitoids evolve a fecundity that matches the number of hosts they are likely to encounter in their lifetime (Godfray, 1994). Eggs of *P. piceae* are usually found in high numbers in mature, isolated fir trees. Once a *Eubazus* sp. female has located an attacked tree, she has the opportunity to meet hundreds of host eggs at the same site. Thus, the fitness of *Eubazus* sp. is maximized by a greater fecundity. In contrast, the spatial distribution of the other *Pissodes* hosts is more scattered because a cone, a leader or a young tree is likely to contain less hosts than a mature fir. Thus, compared to *Eubazus* sp., *E. robustus*, *E. crassigaster* and *E. semirugosus* will probably encounter less hosts in their lifetime. For these species, the number of hosts parasitized will largely depend on their searching efficiency. As a result, instead of allocating resources to increased fecundity, they probably allocate resources to increased searching and flying capacity.

There is no reason to believe that, in *E. semirugosus* and *E. robustus*, the mountain, diapausing biotype and the lowland, non-diapausing biotype are different species. The two biotypes of *E. semirugosus* mate and interbreed as well as specimens from the same biotype, and there are no morphometric or enzymatic characters which separate them. Diapause is merely an adaptation to synchronize their phenology to that of their hosts in different environments (Kenis 1994; Kenis *et al.*, 1996). Nevertheless, diapause in *Eubazus* spp. is genetically based, as it can be transmitted from father to offspring. This observation shows the potential of hybridization in the improvement of *Eubazus* parasitoids and of biological control agents in general. The diapausing biotype of *E. semirugosus* was selected as the best agent for the biological control of the white pine weevil, because the diapause characteristic allows the parasitoid to synchronize its life cycle with that of the target host (Kenis, 1994; Kenis *et al.*, 1996). However, this strain might be less effective in other traits, such as host location. Indeed, the diapausing biotype is reared from pine *Pissodes* spp., while most of the damage by the white pine weevil is observed on spruce (Alfaro, 1982; Lavallée & Benoit, 1989). Transmitting the genes responsible for diapause by hybridization into a biotype or species which naturally locates spruce could improve the potential for biological control. Genetic improvement of natural enemies has often been proposed to enhance their effectiveness as biological control agents (DeBach, 1958; Beckendorf & Hoy, 1985; Hoy, 1990), but, until now, the only satisfactorily results were achieved in the improvement of pesticide resistance in phytoseiid mites (Hoy, 1990).

It is not yet possible to be sure of the identity of the *Eubazus* species that attacks *P. harcyniae*, as only morphometric data are available for comparison. Morphometrics suggests that it is *E. semirugosus*, but live material is needed to carry out cross matings, isoenzyme analyses, or host location comparisons.

Eubazus crassigaster is morphometrically very similar to *E. semirugosus*, but the presence or absence of a tooth on the clypeus separates most individuals, and for the doubtful specimens, separation can be achieved using multivariate discriminant functions. Should *E. semirugosus* be introduced into North America for the biological control of *P. strobi*, the best method to distinguish the two parasitoid species is electrophoresis of the PGDH enzyme, as it provides total separation and has the added advantage of detecting natural hybridization between the native and the exotic species. Nonetheless, additional analyses of different populations of *E. crassigaster* should be made to be sure that the European allele is not present in North America.

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References

Achterberg, C. van (1988) Revision of the subfamily Blacinae Foerster (Hymenoptera, Braconidae). *Zoologische Verhandelingen* **249**, 1–324.

Achterberg, C. van (1990) Revision of the genera *Foersteria* Szépligeti and Polydegmon Foerster (Hymenoptera: Braconidae), with the description of a new genus. *Zoologische Verhandelingen* **257**, 1–32.

Alauzet, C. (1982) Biocenose de *Pissodes notatus* F. ravageur des pins maritimes en forêt de Bouconne (Haute-Garone: France). *Nouvelle Revue d'Entomologie* **12**, 81–89.

Alfaro, R.I. (1982) Fifty-year-old Sitka spruce plantations with a history of intense weevil attack. *Journal of the Entomological Society of British Columbia* **79**, 62–65.

Annila, E. (1975) The biology of *Pissodes validirostris* Gyll. (Col., Curculionidae) and its harmfulness, especially in Scots pine seed orchards. *Communicationes Instituti Forestalis Fenniae* **85**, 1–95.

Beckendorf, S.K. & Hoy, M.A. (1985) Genetic improvement of arthropod natural enemies through selection, hybridization or genetic engineering techniques. pp. 167–187 *in* Hoy, M.A & Herzog, D.C. (*Eds*) *Biological control in agricultural IPM systems*. Orlando, Academic Press.

Bush, G.L. (1975) Sympatric speciation in phytophagous parasitic insects. pp. 187–207 *in* Price, P.W. (*Ed.*) *Evolutionary strategies of parasitoids*. New York, Plenum.

Caltagirone, L.E. (1985) Identifying and discriminating among biotypes of parasites and predators. pp. 189–200 *in* Hoy, M.A & Herzog, D.C. (*Eds*) *Biological control in agricultural IPM systems*. Orlando, Academic Press.

DeBach, P. (1958) Selective breeding to improve adaptations of parasitic insects. *Proceedings of the X International Congress of Entomology 4*, 759.

DeBach, P. & Rosen, D. (1991) *Biological control by natural enemies*. 2nd edn. 440 pp. Cambridge University Press.

Diehl, S.R. & Bush, G.L. (1984) An evolutionary and applied perspective of insect biotypes. *Annual Review of Entomology* **29**, 471–504.

Ehler, L.E. (1990) Introduction strategies in biological control of insects. pp. 111–134 *in* Mackauer, M., Ehler, L.E. & Roland, J. (*Eds*) *Critical issues in biological control*. Andover, Intercept.

Fahringer, J. (1934) *Opuscula Braconologia, palaearktische region Bd. 2.* pp. 321–594. Wien.

Gauld, I.D. (1986) Taxonomy, its limitations and its role in understanding parasitoid biology. pp. 1–21 *in* Waage, J. & Greathead, D.J. (*Eds*) *Insect parasitoids*. London, Academic Press.

Godfray, H.C.J. (1994) *Parasitoids. Behavioral and evolutionary ecology*. 473 pp. Princeton University Press.

Haeselbarth, E. (1962) Zur Biologie, Entwicklungsgeschichte und Oekologie von *Brachistes atricornis* Ratz. als eines Parasiter von *Pissodes piceae*. *Zeitschrift für Angewandte Entomologie* **49**, 233–289.

Holler, C. (1991) Evidence for the existence of a species closely related to the cereal aphid parasitoid *Aphidius rhopalosiphi* De Stefani-Perez based on host ranges, morphological characters, isoelectric focusing banding patterns, cross-breeding experiments and sex pheromone specificities (Hymenoptera, Braconidae, Aphidiinae). *Systematic Entomology* **16**, 15–28.

Hopper, K.R., Roush, R.T. & Powell, W. (1993) Management of genetics of biological control introductions. *Annual Review of Entomology* **38**, 27–51.

Hoy, M.A. (1990) Genetic improvement of arthropod natural enemies: becoming a conventional tactic? pp. 405–415 *in* Baker, R.R. & Dunn, P.E. (*Eds*) *New directions in biological control*. New York, Alan R. Lyss.

Kenis, M. (1994) Variations in diapause among populations of *Eubazus semirugosus* (Nees) (Hym.: Braconidae), a parasitoid of *Pissodes* spp. (Col.: Curculionidae). *Norwegian Journal of Agricultural Sciences, Supplement* **16**, 77–82.

Kenis, M. & Mills, N.J. (1994) Parasitoids of European species of the genus *Pissodes* (Col: Curculionidae) and their potential for the biological control of *Pissodes strobi* (Peck) in Canada. *Biological Control* **4**, 14–21.

Kenis, M., Hulme, M.A. & Mills, N.J. (1996) Comparative developmental biology of populations of three European and one North American *Eubazus* spp. (Hymenoptera: Braconidae), parasitoids of *Pissodes* spp. weevils. *Bulletin of Entomological Research* **86**, 143–153.

Kishi, Y. (1970) Difference in sex ratio of the pine bark weevil parasite, *Dolichomitus* sp. (Hymenoptera: Ichneumonidae) emerging from different host species. *Applied Entomology and Zoology* **5**, 126–132.

Kudela, M. (1974) Curculionidae, Pissodini. pp. 299–310 *in* Schwenke, W. (*Ed.*) *Die Forstschädlinge Europas 2 Band*. Hamburg, Paul Pareys.

Kudon, L.H. & Berisford, C.W. (1980) Influence of brood hosts on host preference of bark beetle parasites. *Nature* **283**, 288–289.

Laidlaw, W.B.R. (1933) Two British parasites of *Pissodes*. *Scottish Forestry Journal* **47**, 24–31.

Lavallée, R. & Benoit, P. (1989) Le Charançon du pin Blanc. *Forêts Canada, Feuillet d'information*, CLF **18**, 1–13.

Lovaszy, P. (1941) Zur Kenntnis der Schlupwespen einiger schädlicher Rüsselkäfer. *Annales Entomologica Fennici* **7**, 194–204.

Marshall (1888) *Species des hymenoptères d'Europe et d'Algérie, tome quatrième*. 609 pp. Beaune (France).

Marshall (1891) *Species des hymenoptères d'Europe et d'Algérie, tome cinquième*. 635 pp. Gray (France), Bouffaut Frères.

Mayr, E. (1942) *Systematics and the origin of species, from the viewpoint of a zoologist*. New York, Columbia University Press.

Mendel, Z. (1986) Hymenopterous parasitoids of bark beetles (Scolytidae) in Israel: relationships between host and parasitoid size, and sex ratio. *Entomophaga* **31**, 127–137.

Mills, N.J. & Fisher, P. (1986) The entomophage complex of *Pissodes* weevils, with emphasis on the value of *P. validirostris* as a source of parasitoids for use in biological control. pp. 297–305 *in* Roques A. (*Ed.*) *Proceedings of the 2nd International Conference IUFRO Cone and Seed Insects Working Party, Briançon, September 1986*. Olivet (France), INRA.

Murphy, R.W., Sites, J.W., Buth, D.G. & Haufler, C.H. (1990) Proteins 1: isozyme electrophoresis. pp. 45–126 *in* Hillis, D.M. & Moritz, C. (*Eds*) *Molecular systematics*. Sunderland, Massachussets, Sinauer.

O'Brien, C.W. (1989a) Revision of the weevil genus *Pissodes* in Mexico with notes on the neotropical Pissodini (Coleoptera, Curculionidae). *Transactions of the American Entomological Society* **115**, 415–432.

O'Brien, L.B. (1989b) A catalog of the Coleoptera of America north of Mexico. Family Curculionidae, subfamily Pissodinae Bedell 1888. *Agriculture Handbooks*. **529–143d**, 1–8.

Powell, W. & Walton, M.P. (1989) The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pests. pp. 343–365 *in* Loxdale, H.D. & Den Hollander, J. (*Eds*) *Electrophoretic studies on agricultural pests*. Oxford, Clarendon Press.

Price, P.W. (1975) Reproductive strategies of parasitoids. pp. 87–111 *in* Price, P.W. (*Ed.*) *Evolutionary strategies of parasitoids*. New York, Plenum.

Ratzeburg, J.T.C. (1844) *Die Ichneumonen der Forstinsekten, Bd. 1*. 226 pp. Berlin.

Ratzeburg, J.T.C. (1848) *Die Ichneumonen der Forstinsekten, Bd. 2*. 238 pp. Berlin.

Reyment, R.A., Blackith, R.E. & Campbell, N.A. (1984) *Multivariate morphometrics*. 233 pp. London, Academic Press.

Roques, A. (1975) *Etude de la mérocénose des cônes de pins sylvestres en forêt de Fontainebleau*. 164 pp. Thèse 3e cycle, Paris VI.

SPSS (1993) *SPSS for Windows, advanced statistics, Release 6.0*. 578 pp. Chicago, SPSS Inc.

Turlings, T.C.J., Wäckers, F.L., Vet, L.E.M., Lewis, W.J. & Tumlinson, J.H. (1993) Learning of host-finding cues by hymenopterous parasitoids. pp. 51–78 *in* Lewis, A.C. & Papaj, D.R. (*Eds*) *Insects learning: ecological and evolutionary perspectives*. New York, Chapman and Hall.

Vet, L.E.M., Janse, C.J., van Achterberg, C. & van Alphen, J.J.M. (1984) Microhabitat location and niche segregation in two sibling species of drosophilid parasitoids: *Asobara tabida* (Nees) and *A. rufescens* (Foerster) (Braconidae: Alysiinae). *Oecologia* **61**, 182–188.

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